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APPLICATION NUMBER: ***60/555,125***
FILING DATE: ***March 22, 2004***
RELATED PCT APPLICATION NUMBER: ***PCT/US05/09358***

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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17510 U.S. PTO
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032204

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
EDUARDO	MARBAN	LUTHERVILLE, MD

Additional inventors are being named on the ______ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

PRE-TRANSPLANTATION MODIFICATION OF CELLS TO IMPROVE ELECTRICAL AND CONTRACTILE FUNCTION

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS ***(check all that apply)***

☑ Specification *Number of Pages* 7

☐ Drawing(s) *Number of Sheets* ______

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number ______

☐ Other (specify) ______

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Respectfully submitted,

SIGNATURE [signature]

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Date 3/22/04

REGISTRATION NO. 45,282
(if appropriate)
Docket Number: 4427

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PROVISIONAL APPLICATION COVER SHEET
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PTO/SB/16 (08-03)
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Docket Number	4427

INVENTOR(S)/APPLICANT(S)		
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Number __________ of__________

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U.S. Provisional Patent Application

JHU Ref. No. DM-4427

Pre-Transplantation Modification of Cells to Improve Electrical and Contractile Function

Inventors: Drs. Eduardo Marban and Maria Abraham

All publications, patents and patent applications disclosed herein are incorporated into this application by reference in their entirety.

a) *General purpose:* To improve the electrical and contractile function of hearts undergoing cell transplantation with stem cells or myoblasts.

b) *Background:* Heart failure (HF) is a major public health problem in the United States. Despite great strides in the management of HF, it is still associated with high mortality and morbidity. Cardiac transplantation is the only definitive treatment for end stage heart failure, but is limited by the availability of organs, which has led to a great deal of interest in cellular cardiomyoplasty. The general goal of such therapy is to improve cardiac function by seeding the heart with exogenous cells. The hope is that such transplanted cells will function within the heart as contracting, electrically-excitable cells. The problem is that such cells, when implanted, may not become part of the electrical syncytium of the heart. Instead, they may create islands of non-connected tissue that disrupt normal electrical conduction to produce arrhythmias. Electrically-isolated cells also may undermine rather than improve contractile function.

We have developed an invention that "improves" cells prior to transplantation, so that they end up being better-coupled to the cardiac syncytitium. The example here, which has been reduced to practice, is the genetic modification of skeletal myoblasts with genes that improve electrical coupling (connexins). However, such pretreatment can be generalized to any cells destined for transplantation. Such genetic modification may also be important for cells that will be transplanted into other organs (e.g., skeletal muscle, uterus, brain).

Skeletal muscle has the capacity to regenerate because of satellite cells that provide a source of cells for cardiac repair. Injection of myoblasts derived from skeletal muscle satellite cells has been shown to improve cardiac performance in phase 1 studies of patients with HF. However, the enthusiasm for myoblast transplantation has been tempered by the high incidence of ventricular tachycardia observed in patients (10 of the first 22 reported cases) after myoblast transplantation. Until now, the mechanisms of arrhythmias induced by myoblast transplantation have remained an enigma, possibly because there have been no reproducible models of such arrhythmias. We have now successfully reproduced reentrant arrhythmias by co-culturing myoblasts and neonatal rat ventricular myocytes (NRVMs) in an in vitro (monolayer) system. Myoblast-myocyte co-cultures are arrhythmogenic due to lack of coupling between them. This results in wavebreaks and reentry.

c) *Description and operation:* In general, somatic gene transfer methods are used to introduce genes into the cells that will be transplanted, prior to transplantation. Ideally such genetic modification will be stable. Here, a lentivirus is used to transduce myoblasts with the Cx43 or Cx40 genes resulting in the production of a stable cell line expressing the above. Coupling of connexin-expressing myotubes with NRVMs decreases the propensity for arrhythmias. Since Cx40 has a higher single channel conductance than Cx43, Cx40 transduction may enhance cell-cell coupling to a greater extent than Cx43. Myoblasts transduced with the connexins can be amplified and injected at the time of cardiomyoplasty.

LV-Cx43 and LV-CX40 have been generated . Expression was confirmed by fluorescence microscopy, (Fig 1) immunostaining and western blot.

LV-Cx43 and LV-Cx40 resulted in a transduction efficiency of 80-90%. Transduction resulted in stable gene expression over 6 weeks in culture. Cx43 transduction resulted in decreased cell proliferation compared to non-transfected and GFP-transfected myoblasts. Also, intensity of GFP fluorescence was less in LV-Cx43 and LV-Cx40 transduced myoblasts when compared to LV-GFP transduced myoblasts. This can be attributed to the fact that the gene encoding GFP was subcloned after the IRES (internal ribosomal entry site).

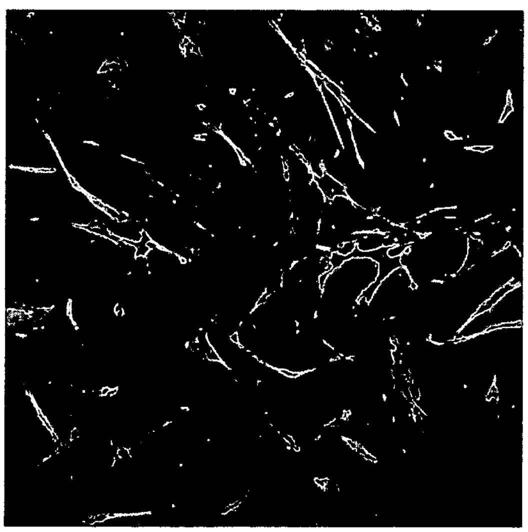

Fig 1: Fluorescence microscopy of LV-Cx43 transduced myoblasts

Preliminary Data

Myoblasts differentiate into myotubes that lack connexins resulting in decreased overall coupling when mixed with myocytes. In order to study myotube-myocyte interactions, myoblasts transduced with GFP or Cx43 or Cx40 were co-cultured and then subjected to optical mapping, calcium imaging and immunostaining.

1. Examination of co-cultures revealed synchronous beating of the monolayer, due to contraction of the NRVMs. Individual myotubes (identified by GFP) did not exhibit contractions, and showed random orientation.

2. NRVM-myoblast (GFP only) co-cultures (ratio of 8:2) demonstrated severe decrease in conduction velocity. (5.2 cm/s, n=2 in co-cultures; mean 22, n=2 in NRVM-only controls). (fig2)

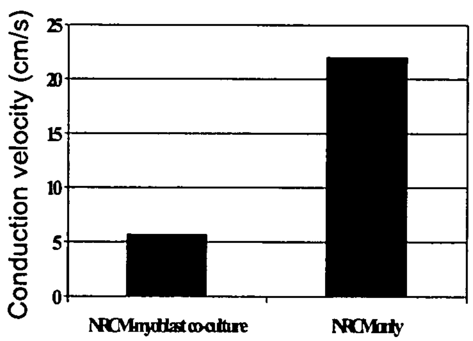

Fig 2. Conduction velocity in seven co-cultures of myoblasts and myocytes (NRCM) and controls (NRCM only).

3. Reentrant arrhythmias were observed spontaneously (n=1) and after pacing in all NRVM-myoblast (GFP) co-cultures that were optically mapped and in none of the controls. This reproduces in-vitro, the increased incidence of reentrant ventricular arrhythmias observed in patients receiving myoblast transplants. Fig 3 is an example of a single rotor that was present spontaneously (before pacing was begun). The color bar in the figure corresponds to normalized voltage level, with blue being the resting state and red being peak of action potential.

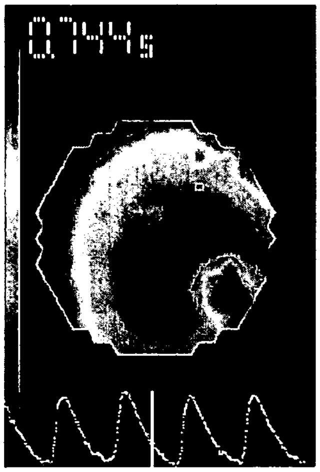

Fig 3. Optical action potentials and voltage map of reentry in a myoblast-NRVM co-culture.

4. Connexin 43 transduction of the myoblasts increased conduction velocity and prevented initiation of reentry.

8. **Unique aspects of this technology.**

- A method, based upon gene transfer into cells destined for transplantation, to improve the electrical and contractile function of hearts.
- A method, based upon gene transfer into myoblasts, to create electrically coupled myotubes.
- The use of said method to increase coupling between cardiac myocytes and myoblasts/myotubes.
- The use of said method, in conjunction with cellular transplantation, to reduce the arrhythmogenic potential of myoblast transplantation in hearts.
- The use of said method, in conjunction with cellular transplantation, to increase electromechanical coordination and thereby reduce the arrhythmogenic potential of cell transplantation.
- The use of said method, in conjunction with cellular transplantation, to increase electromechanical coordination and thereby to improve mechanical and contractile function of the heart after cell transplantation

9. **Publications and planned disclosures.**

A disclosure of the general concept is planned at a public meeting in May, 2004, of the NASPE-Heart Rhythm Society. Abstracts are attached.

10. **Sale or public use:** None.

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Activity : Abstract Submission
Current Date/Time : 12/5/2003 7:48:19 AM

Mixtures of neonatal rat cardiomyocytes and skeletal muscle myoblasts form an arrhythmic substrate

Charles A. Henrikson, MD, Barun Maskara, BS, Leslie Tung, PhD, Miguel Aon, PhD, Felipe Aguel, PhD, Sean Sheehy, BS, Tian Xue, Ronald A. Li, PhD, Eduardo Marban, MD, PhD and M. Roselle Abraham, MD. Johns Hopkins Division of Cardiology, Baltimore, MD, Johns Hopkins Department of Biomedical Engineering, Baltimore, MD

Cellular myoplasty of the heart is a novel therapy for ischemic cardiomyopathy. Recent clinical trials, however, have shown a tendency towards arrhythmogenesis in patients undergoing transplantation of skeletal muscle myoblasts (SkMM). We examined coupling of SkMM with NRCMs using optical mapping and 2-photon microscopy. SkMM and neonatal rat cardiomyocytes (NRCM) were co-cultured on 20 mm coverslips (n=7), where NRCMs were plated on half of the coverslip, and SkMMs transfected with lentivirus expressing GFP were plated 24 hrs later. Electrical activity was optically mapped using the voltage-sensitive dye di-4-ANEPPS. Six days after the second plating, stimulation in the NRCM half of the monolayer revealed prolongation of the action potential and slowed conduction in the NRCM half, and lack of propagation into the myoblast half. Stimulation in the myoblast half of the monolayer did not produce propagated wavefronts. Fluorescence microscopy demonstrated that SkMMs not only covered half of the cover slip, but were also interposed in between the NRCMs. In another series of experiments, SkMMs were co-cultured with NRCMs as mixed monolayers. Optical mapping revealed severe decrease in conduction velocity (5.7+/- 2.1 cm/s, n=7 in co-cultures; mean 25 cm/s, n=2 in NRCM-only controls) and marked prolongation in APD80 (197+/-17 ms in co-cultures; 92 ms, n=2 in controls). Calcium imaging by 2-photon microscopy using Indo-1-AM revealed poor cell-to-cell coupling between SkMMs and NRCMs. We conclude that NRCMs grown in the presence of SkMMs have impaired conduction and prolonged APD80, and that little functional coupling occurs between NRCMs and SkMMs. These observations suggest that mixtures of NRCMs and SkMMs can form an arrhythmic substrate, and may offer insight into the increased clinical arrhythmogenesis observed in patients undergoing SkMM transplant following myocardial infarction.
Author Disclosure Block: **C.A. Henrikson**, None; **B. Maskara**, None; **L. Tung**, None; **M. Aon**, None; **F. Aguel**, None; **S. Sheehy**, None; **T. Xue**, None; **R.A. Li**, None; **E. Marban**, None; **M. Abraham**, None.

Category (Complete): 3. Cell Physiology, Pharmacology, and Signaling
Keyword (Complete): Electrophysiology
Additional Abstract Information (Complete):
  Presentation Type : None
  I am eligible for the Young Investigators Award: : No
  I am eligible for an abstract award: : Fellows
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Sustained reentry in co-cultured monolayers of skeletal muscle myoblasts and neonatal rat cardiomyocytes

M. Roselle Abraham, MD, Charles A. Henrikson, MD, Barun Maskara, BS, Felipe Aguel, PhD, Sean Sheehy, BS, Tian Xue, Ronald Li, PhD, Miguel Aon, PhD, Eduardo Marban, MD, PhD and Leslie Tung, PhD. Johns Hopkins Division of Cardiology, Baltimore, MD, Johns Hopkins Department of Biomedical Engineering, Baltimore, MD

Recent clinical trials of skeletal muscle myoblasts (SkMM) for cardiomyoplasty following MI have shown a high incidence of malignant ventricular arrhythmias. We investigated possible cellular mechanisms of SkMM-induced arrhythmia by co-culturing SkMM transduced with a lentivirus expressing GFP with neonatal rat ventricular myocytes (NRVM). SkMMs and NRVMs were grown as a mixed monolayer on 20 mm coverslips in cell ratios of 1:9 or 2:8. Light microscopy demonstrated a relatively uniform distribution of myoblasts and myocytes. Electrical activity was optically mapped using the voltage-sensitive dye di-4-ANEPPS. Cell monolayers were paced with an 8 pulse train at 1 to 5 Hz. In all co-cultured monolayers (n=7), the depolarization wavefront was irregular and wavebreaks occurred even at low pacing rates; conduction block developed between 4.4 and 5Hz, and reentry could be induced. These effects were absent in NRVM-only controls (n=7). Reentrant activity was varied, and consisted of single or figure-of-eight rotors that were drifting, transient, or stable. Average reentry cycle length was 214 ms (n=7) in the co-cultures, compared with cycle lengths of 100ms (n=2) in NRVM-only monolayers. Two co-cultures had rotors that were stable for >60 minutes, while in one co-culture, a stable rotor was present prior to initiating the pacing protocol. Nitrendipine (10 μM) slowed conduction velocity and terminated the reentrant arrhythmias. In summary, we have demonstrated the presence of readily-induced , sustained reentrant activity in co-cultures of SkMM and NRVM.
Author Disclosure Block: **M. Abraham,** None; **C.A. Henrikson,** None; **B. Maskara,** None; **F. Aguel,** None; **S. Sheehy,** None; **T. Xue,** None; **R. Li,** None; **M. Aon,** None; **E. Marban,** None; **L. Tung,** None.

Category (Complete): 3. Cell Physiology, Pharmacology, and Signaling
Keyword (Complete): Arrhythmias - mechanism ; Reentry
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